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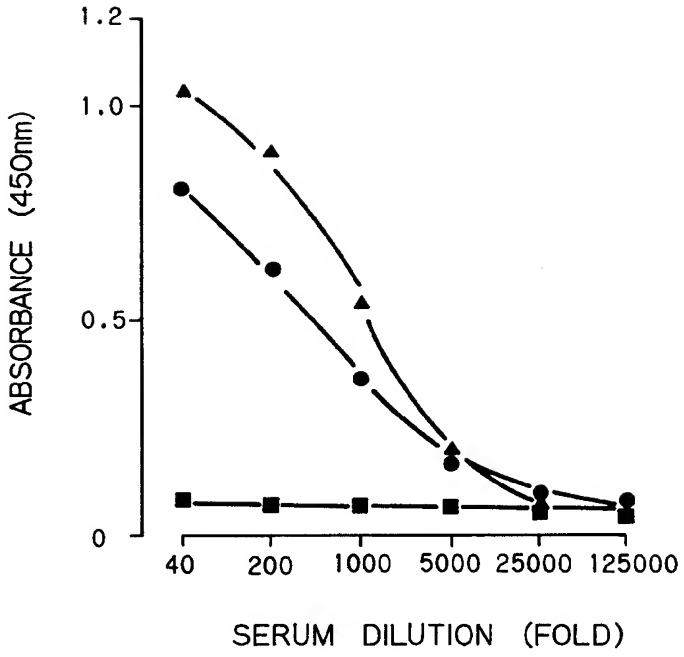
(54) **SOLID-PHASE REAGENT AND ASSAY OF ANTIBODY USING THE SAME.**

(57) An antibody specific for an antiphospholipid antibody syndrome can be specifically assayed by using a solid-phase reagent prepared by binding β2-glycoprotein I to a carrier having a functional group and/or a free radical with a negative charge or a lone electron pair attached to the surface thereof. Also an antibody specific for an antiphospholipid antibody syndrome and another antibody specific for an infectious disease can be differentially detected by using the above reagent or a modification thereof.

EP 0 600 088 A1

FIG. 1


1.2
1.0
0.5
0
ABSORBANCE (450nm)
40
200
1000
5000
25000
125000
SERUM DILUTION (FOLD)

FIELD OF THE INVENTION

The present invention relates to a solid phase reagent obtained by binding β2-glycoprotein I to a specific carrier, an assay method for antibodies specific to antiphospholipid syndrome using the solid phase reagent, a method for detecting antibodies specific to antiphospholipid syndrome and antibodies specific to infectious diseases differentially from each other, and a kit for use in the method.

BACKGROUND

Various assay methods including RIA and ELISA for anticardiolipin antibodies which are one of antiphospholipid family had been reported by Harris et al. (Lancet, iii: 1211, 1983), by Koike et al. (Clin. Exp. Immunol., 56: 193, 1984), etc.

However, those methods described above are not necessarily satisfactory since they involve problems that the anticardiolipin antibodies cannot be assayed quantitatively or antibodies associated with infectious diseases cannot be assayed differentially from antibodies found in patient with antiphospholipid syndrome.

Recently, Matsuura et al. found that anticardiolipin antibodies associated with antiphospholipid syndrome does not recognize an immobilized cardiolipin but the complex of cardiolipin and β2-glycoprotein I (alternatively termed apolipoprotein H or anticardiolipin cofactor). Further, they developed an assay method for antiphospholipid antibody determination based on the new findings described above, which can overcome the prior art problems as described above (Lancet, 336: 177, 1990, RINSHO MEN-EKI (Clinical Immunology), 22 (Suppl. 15): 170, 1990, WO 91/06006, J. Immunol., 148:3855, 1992).

The studies by Matsuura et al. revealed that anticardiolipin antibodies associated with antiphospholipid syndrome recognize the complex of cardiolipin and β2-glycoprotein I. However, clinical significance of the antibodies has been still unclear. Under the circumstance, clarifying the clinical significance on anticardiolipin antibody binding should elucidate pathogenesis of antiphospholipid syndrome. This is an important issue to be focused in the future.

To solve the problems, it is important to develop a more convenient assay method having high specificity and quantitative property. However, according to the conventional RIA and ELISA, it was impossible to detect differentially the antibodies directed to the complex of cardiolipin and β2-glycoprotein I which is found in the patient with antiphospholipid syndrome from those directed to cardiolipin associated with infectious diseases such as syphilis, as described above. Although, an assay method improved by Matsuura et al. could provide differential detection of those antibodies, the preparation of solid phase reagents was still complicated on the other hand. It has thus been desired to establish a more convenient assay method.

Accordingly, an object of the present invention is to provide an assay method in a simpler manner for antibodies specific to the complex of cardiolipin and β2-glycoprotein I.

DISCLOSURE OF THE INVENTION

As a result of extensive studies to achieve the object described above, the present inventors have found that antibodies specific to antiphospholipid syndrome can be detected by using as a solid phase reagent a β2-glycoprotein I-coated carrier of which surface is bearing functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair. The present invention has thus been accomplished.

Accordingly, the present invention relates to a solid phase reagent comprising a β2-glycoprotein I-coated carrier of which surface is bearing functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair (hereinafter sometimes simply referred to as solid phase reagent I).

The present invention also relates to an assay method by using the solid phase reagent I for antibodies specific to antiphospholipid syndrome and a kit for use in the method.

The present invention further relates to an assay method for differentially detecting antibodies specific to an antiphospholipid syndrome from those specific to infectious diseases and a kit for use in the method.

The present invention further relates to a solid phase reagent for use in the aforesaid differential assay method, which reagent comprises a carrier having the surface onto which functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair have been previously introduced and having two sites, one of which being a site on which β2-glycoprotein I has been coated and another being a site on which no β2-glycoprotein I has been coated (hereinafter sometimes simply referred to as solid phase reagent II).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results obtained by conventional ELISA.

Fig. 2 shows the results obtained by the improved method by Matsuura et al.

Fig. 3 shows the results obtained by the method of the present invention.

Fig. 4 indicates correlation between the results obtained by the method of the present invention and those obtained by the improved method of Matsuura et al.

Fig. 5 shows the reactivity of anticardiolipin antibodies with β2-GPI-coated plates which were previously irradiated with radiation or electron beam.

Fig. 6 shows specificity of monoclonal antibody WB-CAL-1.

Fig. 7 shows specificity of serum As.

Fig. 8 shows specificity of anti-β2-GPI antibody (Cof-18).

Fig. 9 shows the results of anticardiolipin determination using carboxylated plates.

Fig. 10 shows correlation between anticardiolipin antibody titers obtained using plates irradiated with radiation and those obtained using CL-coated plates.

Fig. 11 shows correlation between anticardiolipin antibody titers obtained using carboxylated plates and those obtained using CL-coated plates.

Fig. 12 shows correlation between anticardiolipin antibody titers obtained using plates irradiated with radiation and those obtained using carboxylated plates.

Fig. 13 shows the results of anticardiolipin determination using commercially available polystyrene plates on which β2-GPI has been previously coated.

Fig. 14 shows the results of differential assay for anticardiolipin antibodies associated with autoimmune and infectious diseases.

Fig. 15 shows the results of surface analysis of polystyrene plates of the present invention which were previously irradiated with radiation or electron by X-ray photoelectron spectroscopy.

BEST MODE FOR PRACTICING THE INVENTION

Hereinafter the present invention is described in more detail.

(1) Solid phase reagent of the present invention

The solid phase reagent I of the present invention comprises a β2-glycoprotein I-coated carrier of which surface is bearing functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair.

The solid phase reagent I can be used in the assay method for antibodies specific to an antiphospholipid syndrome and used in the kit therefor.

In the present specification, the term "antiphospholipid syndrome" is used to mean autoimmune disease including representatively systemic lupus erythematodes (SLE), or a group of diseases showing symptoms such as in thrombosis, neuropathy, recurrent abortion, thrombothytopenia, etc. (J. Rheumatol., 13, 486 (1986)).

As the carrier used to coat β2-glycoprotein I thereto, any types of carrier can be used without any particular restriction so long as the carrier has a surface onto which functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair have been previously introduced.

Herein, the functional group containing a negative charge or a lone pair and/or free radical containing a negative charge or a lone pair refers to those containing a negative charge or a lone pair in the molecule thereof. Specific examples include functional groups such as hydroxy, carboxyl, carbonyl, formyl, imino, nitro, thiol, sulfonyl, etc., and free radicals such as oxygen radicals, etc. Preferred are the functional groups or radicals in which the negative charge or lone pair is associated with oxygen atom, for example, hydroxy, carboxyl, carbonyl, oxygen radicals, etc. The carrier having a surface which is bearing functional groups and/or free radicals can be prepared by introducing the functional groups containing a negative charge or a lone pair, directly or if necessary chemically, into a synthetic resin having a highly protein-adsorbable hydrophobic surface, more specifically, a synthetic resin such as polyvinyl chloride, polystyrene, styrene-divinylbenzene copolymer, styrene-maleic anhydride copolymer, nylon, polyacrylamide, polyacrylonitrile, polypropylene, polymethylene methacrylate, etc.

For preparing carriers preferably used in the present invention, the following methods can also be used: a method which comprises exposing the aforesaid synthetic resins having a hydrophobic surface to UV ray,

radiations (x ray, β ray, γ ray, etc.), electron beams, or the like to introduce the functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair onto the hydrophobic surface; a method which comprises treating the aforesaid synthetic resins having a hydrophobic surface with ozon, plasma, etc. to introduce the functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair onto the hydrophobic surface; etc.

Such various treatments can be carried out in a conventional manner. For example, in the case that radiations or electron beams are used, the synthetic resins described above are irradiated with approximately 1 to 200 kGray radiation to introduce the functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair onto the hydrophobic surface of the carrier.

Specific examples of such a carrier include EB Plate (manufactured by Labsystems Co., Ltd.), H Type Plate and C Type Plate (manufactured by Sumitomo Bakelite Co., Ltd.), Maxi-Soap Plate (manufactured by Nunc Co., Ltd.), etc.

The carrier can take any one of shapes, such as plate-like type (microtiter plate, disk, etc.), granular type (beads, etc.), tubular type (test tube, etc.), fibrous type, membrane-like type, fine particulate type (latex particles, etc.) and the like. The carrier having an appropriate shape may be chosen depending upon the assay method.

As β2-glycoprotein I which is coated to the carrier, there is no particular restriction so long as it is derived from animal. Especially, β2-glycoprotein I derived from human origin is preferred. β2-Glycoprotein I from which sugar chain has been removed partially or wholly may also be used in the present invention.

β2-Glycoprotein I can be prepared in a conventional manner, for example, by the method of McNeil (Proc. Natl. Acad. Sci. USA, 87: 4120, 1990). Furthermore, the amino acid and nucleotide sequences of β2-glycoprotein I have been already clarified. Thus, β2-glycoprotein I prepared by a conventional peptide synthesis method or DNA recombinant technique can be also used for the present invention.

It is preferred that β2-glycoprotein I be highly purified but this is not so strictly required, either.

β2-Glycoprotein I may be bound to the carrier described above by appropriately combining and conducting conventional processes under suitable conditions known for immobilization of protein such as enzyme (cf., KOTEIKA KOSO (Immobilized Enzyme), 1975, edited by Chihata Ichiro, published by Kodansha, SEIKAKAGU JIKKEN KOZA (Series of Biochemical Experiments) 11; Enzyme immunoassay, 1989, published by Tokyo Kagaku Dojin Publishing Co., etc.). For example, any technique of physical adsorption, ionic bond, covalent bond, etc. may be used.

The solid phase reagent II of the present invention contains two (2) sites on a carrier, one of which is coated with β2-glycoprotein I and another is without β2-glycoprotein I, and the surface of the carrier has functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair previously introduced thereon. The solid phase reagent II is used to detect antibodies specific to antiphospholipid syndrome differentially from those specific to infectious diseases.

Any of the carrier used for the solid phase reagent II, β2-glycoprotein I and the preparation of the solid phase reagent using these materials are basically the same as in the case of the solid phase reagent I described above, and may be appropriately modified in order to be more suitable for the differential assay according to the present invention.

(2) Method of the present invention and the kit therefor

In the present invention, the assay method for antibodies specific to antiphospholipid syndrome is characterized by using the solid phase reagent I of the present invention described above. So long as the solid phase reagent I is used in the method, the determination principle, conditions, etc. for assay are not particularly restricted.

As classification in terms of reaction method, for example, there are known a competitive reaction method and a non-competitive reaction method. In the present invention, any of these methods can be adopted. Further as classification in terms of detection method, there are known a non-labelling method (nephelometry, etc.) for directly detecting the results of an antigen-antibodies reaction, and a labelling method for detecting the results using any marker. Any of these methods is applicable to the present invention. Furthermore, a heterogeneous method which requires BF separation and a homogeneous method which does not require any BF separation are also known, and any of these methods is applicable to the present invention. That is, any assay method suitable for the purpose of the present invention may be appropriately chosen from these known conventional methods.

Details of these conventional methods are described in, for example, the following articles.

(1) "RADIOIMMUNOASSAY, Second Series" edited by Hiroshi Irie, published May 1, 1979 by Kodansha Publishing Co., Ltd.
(2) "KOSO-MENEKI SOKUTEIHO (ENZYMEIMMUNOASSAY) (second edition)" edited by Eiji Ishikawa et al., published December 15, 1982 by Igaku-Shoin Publishing Co., Ltd.
(3) RINSHO-BYORI (Clinical Pathology), extra special issue No. 53 "Immunoassay for clinical inspection - technique and application -", published by RINSHO BYORI KANKOKAI, 1983
(4) "Dictionary of Biotechnology", published October 9, 1986 by CMC Publishing Co., Ltd.
(5) "Methods in ENZYMOLOGY Vol. 70" (Immunochemical techniques (Part A))
(6) "Methods in ENZYMOLOGY Vol. 73" (Immunochemical techniques (Part B))
(7) "Methods in ENZYMOLOGY Vol. 74" (Immunochemical techniques (Part C))
(8)"Methods in ENZYMOLOGY Vol. 84" (Immunochemical techniques (Part D: Selected Immunoassay))
(9) "Methods in ENZYMOLOGY Vol. 92" (Immunochemical techniques (Part E: Monoclonal Antibodies and General Immunoassay Methods))
(Articles (5)-(9) were published by Academic Press)

The assay method in accordance with the present invention is described below more specifically, taking as one example ELISA in which β2-glycoprotein I-coated microtiter plate having the surface onto which the functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair have been previously introduced is used as a solid phase reagent.

Firstly, a sample solution (e.g., blood, serum, etc.) is added to each well of β2-glycoprotein I-coated plates to react β2-glycoprotein I with antibodies in the sample solution. Next, after washing the wells, enzyme-labelled anti-immunoglobulin antibodies (e.g., peroxidase-labelled anti-IgG antibodies, etc.) is reacted with the resulting complex followed by separation of the solid phase from the liquid phase. After a substrate (in the case of peroxidase, e.g., hydrogen peroxide and tetramethylbenzidine, etc.) is added to the solid phase or the liquid phase, the enzyme activity in either phase is determined. Finally, an amount of the antibodies corresponding to the measurement data obtained is calculated based on a calibration curve previously prepared.

The kit of the present invention for use in the assay method for antibodies specific to antiphospholipid syndrome is also characterized in that the solid phase reagent I of the present invention described above is comprised as a constituent reagent. Other constituent reagents may be appropriately chosen and used in combination so as to be more suitable for the assay method practiced.

For example, the kit for practicing ELISA described above comprises the following reagents.
(1) β2-glycoprotein-coated solid phase reagent;
(2) enzyme-labeled anti-immunoglobulin antibodies;
(3) substrate solution; and,
(4) standard antibodies solution having a known concentration.

Next, differential assay for antibodies specific to antiphospholipid syndrome from antibodies specific to infectious diseases is performed utilizing a reactivity difference between the reaction of the respective antibodies with the site (reagent) to which β2-glycoprotein I has been bound and the reaction of the respective antibodies with the site (reagent) to which no β2-glycoprotein I has been bound. That is, antibodies specific to antiphospholipid syndrome bind specifically to the β2-glycoprotein I-coated site (reagent), whereas antibodies specific to infectious diseases do not show such a tendency. By detecting the reactivity difference, it could be identified from which the antibodies to be analyzed in a sample is derived, so that the respective antibodies can be assayed differentially from each other.

Therefore, the differential assay can be performed in the same manner as in the aforesaid assay method for antibodies specific to antiphospholipid syndrome described above, except for using either the solid phase reagent II described above or combination of two solid phase reagents, i.e., the solid phase reagent I obtained by coating with β2-glycoprotein I the carrier of which surface is bearing the functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair, and the solid phase reagent on which no β2-glycoprotein I has been coated.

The kit for use in the differential assay for antibodies specific to antiphospholipid syndrome and antibodies specific to infectious diseases is also characterized by using either the solid phase reagent II described above or combination of two solid phase reagents, i.e., the solid phase reagent I obtained by coating with β2-glycoprotein I the carrier of which surface is bearing the functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair, and the solid phase reagent which no β2-glycoprotein I has been coated. Other reagents may be appropriately chosen and used in combination so as to be more suitalbe for the differential assay method practiced.

It has been for the first time found by the present inventors that by using only β2-glycoprotein I, antibodies specific to antiphospholipid syndrome can be specifically detected. Accordingly, the present

invention does not require combination of β2-glycoprotein I and phospholipid for detecting antibodies specific to antiphospholipid syndrome, so that the reagent for use in the assay can be prepared in an extremely simple manner. Furthermore, the method of the present invention shows a high correlation with the results obtained by the improved assay method by Matsuura et al. and can thus be a more simpler method which is used instead of the improved method by Matsuura et al.

Further, by using the solid phase reagent comprising a carrier having the surface onto which functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair have been introduced and having a site on which β2-glycoprotein I has been coated and another site on which no β2-glycoprotein I has been coated; or by using the combination of two solid phase reagents, i.e., the solid phase reagent I comprising a β2-glycoprotein I-coated carrier of which surface is bearing the functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair and the solid phase reagent on which no β2-glycoprotein I has been coated,

antibodies specific to antiphospholipid syndrome can be differentially assayed from antibodies specific to infectious diseases.

Hereinafter, the present invention is more specifically described by referring to Comparative Examples and Examples.

Comparative Example 1: Conventional ELISA

An ethanolic solution of 50 μg/ml of bovine heart-derived cardiolipin (manufactured by Sigma) was added to each well of 96-well microtiter plates (polystyrene; manufactured by Labsystems Co., Ltd.) in an amount of 50 μl each/well. Ethanol in the well was evaporated under reduced pressure. After drying, 250 μl of phosphate buffered saline (PBS) containing 10% fetal bovine serum (PBS-FBS) (pH 7.4) was added to the each well. The wells were incubated for an hour at room temperature, and then washed three times with 200 μl of PBS containing 0.05% (V/V) Tween 20 (trademark, manufactured by Kishida Chemical Co., Ltd.) (PBS-Tween).

Next, 100 μl each of serum sample appropriately diluted with PBS-FBS was added to each well and the reaction was carried out at room temperature for an hour, followed by washing three times with 200 μl each of PBS-Tween. Then 100 μl each of horseradish peroxidase-labeled anti-human IgG antibodies was added to each well and the reaction was carried out at room temperature for an hour, followed by washing three times with 200 μl each of PBS-Tween. After 100 μl of substrate solution [0.3 mM 3,3',5,5'-tetramethylbenzidine (TMBZ) solution containing 0.003% hydrogen peroxide] was added to react them at room temperature for 10 minutes, 100 μl of a reaction terminator (2N sulfuric acid) was added to the reaction solution. Absorbance at 450 nm was then measured. The enzyme-labeled antibodies used was obtained by conjugating horseradish peroxidase to mouse monoclonal anti-human IgG antibodies (G-O2, IgG class, manufactured by Yamasa Corporation) according to the periodic acid crosslinking method.

The results are shown in Fig. 1, wherein circle, triangle and square designate serum collected from the patient with typical antiphospholipid syndrome (SLE and recurrent abortion), serum collected from the patient with syphilis and serum collected from healthy donor, respectively. As is clearly noted from Fig. 1, the conventional method fails to quantitatively determine antibodies specific to antiphospholipid syndrome differentially from antibodies specific to infectious diseases.

Comparative Example 2: Improved method by Matsuura et al. (WO 91/06006)

An ethanolic solution of 50 μg/ml of bovine heart-derived cardiolipin (manufactured by Sigma) was added to each well of 96-well microtiter plates (polystyrene; manufactured by Labsystems Co., Ltd.) in an amount of 50 μl each/well. Ethanol in the well was evaporated under reduced pressure. After drying, 200 μl of phosphate buffered saline (PBS) containing 1% purified bovine serum albumin (containing no bovine β2-glycoprotein I) (PBS-pBSA) (pH 7.4) was added to the each well. The wells were incubated for an hour at room temperature, and then washed three times with 250 μl of PBS containing 0.05% (V/V) Tween 20 (trademark, manufactured by Kishida Chemical Co., Ltd.) (PBS-Tween).

Next, 50 μl each/well of purified human β2-glycoprotein I (prepared into 30 μg/ml with PBS-pBSA) was added to each well (50 μl each/well of PBS-pBSA was added to the control group). After allowing to stand for 10 minutes, 50 μl each of serum sample appropriately diluted with PBS-pBSA was added to each well and the reaction was carried out at room temperature for 30 minutes. After washing three times with 200 μl each of PBS-Tween, 100 μl each of horseradish peroxidase-labeled anti-human IgG antibodies was added to each well and the reaction was carried out at room temperature for an hour, followed by washing three times with 200 μl each of PBS-Tween. After 100 μl of substrate solution [0.3 mM 3,3',5,5'-tetramethylben-

zidine (TMBZ) solution containing 0.003% hydrogen peroxide] was added to react them at room temperature for 10 minutes, 100 μl of a reaction terminator (2N sulfuric acid) was added to the reaction solution. Absorbance at 450 nm was then measured.

The results are shown in Fig. 2. In Fig. 2, panel (A) and panel (B) designate the group added with β2-glycoprotein I and the group added with none (control group), respectively. The circle, triangle and square symbols have the same significance as in Fig. 1.

By assaying the control group simultaneously, dependency of antibodies in the sample solution on β2-glycoprotein I can be verified, so that antibodies specific to antiphospholipid syndrome can be assayed differentially from antibodies specific to infectious diseases.

Example 1: Assay for antiphospholipid antibodies using solid phase reagent comprising β2-glycoprotein I-coated polystyrene microtiter plates which were previously irradiated with β ray or electron beams:

Purified human β2-glycoprotein I (10 μg/ml: prepared by 10 mM Hepes (pH 7.4) containing 150 mM NaCl (Hepes)) was added to a polystyrene plate (COMBI PLATE EB (trademark), Labsystems Co., Ltd., Finland) previously irradiated with β ray or electron ray in a volume of 50 μl each/well followed by incubation at 4 °C overnight. After washing three times with 200 μl of PBS-Tween, 100 μl each of serum sample appropriately diluted with Hepes-pBSA was added to each well and allowed to stand at room temperature for 30 minutes. After washing with 200 μl of PBS-Tween three times, 100 μl each of horseradish peroxidase-labeled anti-human IgG antibodies was added to each well and the reaction was carried out at room temperature for an hour. After washing three times with 200 μl each of PBS-Tween, 100 μl of substrate solution [0.3 mM 3,3',5,5'-tetramethylbenzidine (TMBZ) solution containing 0.003% hydrogen peroxide] was added to react them at room temperature for 10 minutes. Then 100 μl of a reaction terminator (2N sulfuric acid) was added to the reaction solution. Absorbance at 450 nm was then measured.

The results are shown in Fig. 3. In Fig. 3, panel (A) and panel (B) designate the results obtained using the polystyrene plate irradiated with β ray or electron beams and the results obtained using a non-irradiated polystyrene plate manufactured by the same company as in the above plate, respectively. The circle, triangle and square symbols have the same significance as in Fig. 1.

As is clearly noted from Fig. 3, the results reveal that antibodies associated with antiphospholipid syndrome can be specifically assayed.

Table 1 described below and Fig. 4 show correlation between the two results which were obtained by assaying antiphospholipid antibody titerss in sera collected from patients with antiphospholipid syndrome (19 cases) and from healthy donors (20 cases) by the two methods, i.e., the method described in Reference Example 2 and the method of the present invention. As the result, extremely high correlation (correlation efficiency: 0.97, N = 39) between the results obtained in the assay by adding β2-glycoprotein I of Reference Example 2 and the results obtained by the method of the present invention. It has thus been verified that the method of the present invention enables to assay for antibodies in the sera to the complex of anticardiolipin and β2-glycoprotein I, as in the method of Reference Example 2.

Table 1

Serum		β2-Glycoprotein I (+)	β2-Glycoprotein I (-)	Method of This Invention
APS	1	16.4	3.0	20.1
	2	37.0	<1.3	69.6
	3	19.4	3.3	5.9
	4	62.0	2.8	22.1
	5	6.3	2.1	9.6
	6	5.8	1.3	9.0
	7	4.6	<1.3	11.2
	8	460.4	8.2	509.2
	9	5.8	<1.3	23.0
	10	8.8	2.3	6.9
	11	2.3	<1.3	5.9
	12	44.2	1.5	58.2
	13	64.2	3.5	18.3
	14	604.8	10.8	582.0
	15	3.7	2.3	35.7
	16	53.3	<1.3	188.8
	17	19.2	<1.3	10.6
	18	606.8	10.0	463.0
	19	108.2	3.6	125.0
Normal	1	<1.3	<1.3	4.9
	2	<1.3	<1.3	3.6
	3	<1.3	<1.3	3.5
	4	2.4	1.5	6.9
	5	<1.3	<1.3	5.6
	6	<1.3	<1.3	5.1
	7	<1.3	<1.3	5.6
	8	<1.3	<1.3	4.9
	9	<1.3	<1.3	4.8
	10	<1.3	<1.3	2.5
	11	<1.3	<1.3	5.3
	12	<1.3	<1.3	4.7
	13	<1.3	<1.3	5.2
	14	<1.3	<1.3	4.2
	15	<1.3	<1.3	4.7
	16	<1.3	1.9	4.8
	17	<1.3	<1.3	5.8
	18	<1.3	<1.3	3.8
	19	<1.3	<1.3	3.5
	20	<1.3	<1.3	4.4

(unit/ml)

APS: patient with antiphospholipid syndrome

Normal: healthy donor

Example 2

Antibodies used herein

WB-CAL-1 and WB-CAL-3:

Monoclonal anticardiolipin antibodies WB-CAL-1 and WB-CAL-3 (both are IgG class) are secreted from hybridomas established by cell fusion of myeloma cells with spleen cells from F1 mouse of NZW mouse and BXSB mouse, respectively. The two antibodies have specificity to the complex of caldiolipin (CL) and β2-glycoprotein I (β2-GPI).

As:

As is serum collected from the anticardiolipin antibody positive patient with antiphospholipid syndrome. The serum is reactive with the complex of CL and β2-GPI.

Cof-18:

Monoclonal antibody Cof-18 is obtained from purified human β2-GPI-immunized BALB/c mouse. The monoclonal antibody shows reactivity with β2-GPI (PCT/JP92/00528).

(A): Reactivity of anticardiolipin antibodies with β2-GPI-coated plate irradiated with radiation:

To each well of a 96-well micro test plate [MS-3496F (S type, manufactured by Sumitomo Bakelite Co., Ltd.)] which had been previously exposed to radiations having different doses [γ ray (100, 50, 25, 12.5, 6.3, 3.1, 1.6 kGray) and β ray (or electron beam) (50, 25 kGray)] was added 50 μl of purified human β2-GPI (10 μg/ml, prepared with 150 mM NaCl-containing 10 mM Hepes (pH 7.4) (Hepes)). The system was allowed to stand at 4 °C overnight. After washing each well three times with 200 μl of phosphate buffered physiological saline containing 0.05% Tween 20 (PBS-Tween), 200 μl of 3% gelatin-PBS was added thereto, and then subjected to a blocking treatment by allowing to stand at room temperature for an hour. After gelatin-PBS was removed, 100 μl each of various anticardiolipin antibodies [WP-CAL-1 (500 ng/ml), WB-CAL-3 (500 ng/ml), serum As (200-fold dilution)] and monoclonal anti-β2-GPI antibodies (Cof-18, 500 ng/ml) were added to the system, respectively, followed by allowing to stand at room temperature for 30 minutes. After washing three times with PBS-Tween, 100 μl each of horseradish peroxidase-labeled anti-mouse IgG antibodies (HRP-amIg) or horseradish peroxidase-labeled anti-human IgG antibodies (HRP-ahIg), each of which had been appropriately diluted, was added to each well and then allowed to stand for 30 minutes. After washing, 100 μl of 0.3 mM tetramethylbenzidine (TMBZ) solution containing 0.003% hydrogen peroxide was added to the mixture. Accurately 10 minutes later, 100 μl of 2N sulfuric acid was added to terminate the reaction. Absorbance was measured at 450 nm.

As shown in Fig. 5, all of the three anticardiolipin antibodies showed the reactivity dependent on doses of radiations (γ ray, β ray) or electron beams. Such dependency was also noted with Cof-18, but significant binding was observed also in the non-exposure group, indicating that the dependency was different from the reaction specificity of the anticardiolipin antibodies.

(B): Specificity of anticardiolipin antibodies

A β2-GPI coated plate was prepared in a manner similar to the above (A). In this inhibition test, four kinds of reagents: CL micelle, β2-GPI solution, CL-coated latex and β2-GPI-bound CL-coated latex were used. The procedures of the preparation are briefly shown below.

CL micelle:

CL micelle was prepared by drying ethanol up from CL ethanol solution (manufactured by Sigma) into a film shape and then dispersing and suspending the film with a vortex and a bath sonicator.

β2-GPI solution:

The solution was prepared by diluting purified human β2-GPI with Hepes buffer.

CL-coated latex:

After colloidal silica was removed from latex beads (6.4 μm, Seradyn Co., Ltd.), Cl was coated thereto.

β2-GPI-bound CL-coated latex:

The β2-GPI-bound CL-coated latex was prepared by incubating CL latex in a β2-GPI solution and removing free β2-GPI by centrifugation.

Monoclonal antibody WB-CAL-1 (500 ng/ml) or serum As (200-fold dilution) was adjusted into the respective concentrations shown in Fig. 6 or Fig. 7, and then added to β2-GPI-coated plate, followed by reacting them at room temperature for 30 minutes. The following procedures for determining the amount of antibodies bound were carried out in the same manner as in the above (A). As shown in Figs. 6 and 7, the binding of anticardiolipin antibodies to the coated β2-GPI occurred only on the β2-GPI-bound CL-coated latex and was inhibited in a dose dependent manner. For information, the results of specificity test of anti-β2-GPI antibodies are shown in Fig. 8. As the result, the binding of anti-β2-GPI antibody (Cof-18) to the coated β2-GPI was inhibited by β2-GPI in a dose dependent manner.

(C): Assay for anticardiolipin antibodies using polystyrene plate onto which carboxyl groups were chemically introduced

β2-GPI was coated to a polystyrene plate onto which carboxyl groups had been chemically introduced (carboxylated plate) in a manner similar to the above (A) and the amount of anticardiolipin antibodies bound was quantitatively determined by the same procedures as in the above (A).

As the result, monoclonal antibodies WB-CAL-1 and WB-CAL-3 showed specific binding to the β2-GPI coated plates, as shown in Fig. 9. Serum collected from healthy donor as control showed no binding under any conditions.

(D): Correlation among anticardiolipin antibody titers obtained using plates irradiated with radiation, carboxylated plates and CL-coated plates

Using plates exposed to γ ray of 100 kGray or carboxylated plates to which β2-GPI had been coated, antibody titers in the APS-derived serum collected from the anticardiolipin antibody positive patient were assayed in the same manner as in the above (A). In addition, the anticardiolipin antibody titer was assayed by the improved assay method by Matsuura et al. (WO91/06006). Correlation in measurement data (absorbance) among the three assay methods was thus examined. As shown in Figs. 10 through 12, a high correlation was noted among the three assay methods.

(E): Assay for anticardiolipin antibodies using β2-GPI coated on commercially available polystyrene plates

The basic procedures for assay are the same as in the above (A). The plates used are Universal Plate (intact plate) and EB Plate by Labsystems Co., Ltd., and S type (intact plate), H type (γ ray-exposed plate) and C type (carboxylated plate) by Sumitomo Bakelite Co., Ltd. As shown in Fig. 13, it became possible to assay for the anticardiolipin antibodies by using the plate obtained by exposure to β ray or γ ray, or by using the plate obtained by introduction of carboxyl groups through chemical modification.

Example 3: Differential assay for respective anticardiolipin antibodies specific to autoimmune and infectious diseases

After 50 μl each of purified β2-GPI (10 μg/ml, prepared with Hepes) was added to each well of a carboxylated plate (C type, manufactured by Sumitomo Bakelite Co., Ltd.), incubation was performed at 4°C overnight. In the control group, Hepes buffer described above was charged. After washing three times with PBS-Tween, gelatin-PBS (200 μl) was added to effect blocking at room temperature for an hour. After gelatin-PBS was removed, 100 μl each of serum sample diluted to 200 fold with Hepes-PBS was added to each well and then settled at room temperature for 30 minutes. Thereafter, the same procedures as in Example 2 (A) were conducted. As shown in Fig. 14, the APS-derived anticardiolipin antibodies showed binding only to the plate coated with β2-GPI, whereas the syphilis-derived antibodies were specifically bound to the carboxylated plates independently on β2-GPI.

Example 4: Analysis of the surface of radiation- or electron beam-exposed polystyrene plate by x-ray photoelectron spectroscopy; XPS

XPS analysis was performed by ESCA Spectrometer (JPS-9000MC, JEOL Ltd., Tokyo Japan). Survey scanning spectrum of 0-1000 ev and C1s spectrum were determined at MgKa 1,2 (1253.6 ev). In this case, the energy passed was 10 eV and resolution was 0.9 ev at the Ag 3d 5/2 peak. Correction was made by the C-C bond energy of the C1s peak as 285.0 ev. For the spectrum analysis, curve fitting of Gaussian/Lorentzian (80 : 20) was used. As shown in Fig. 15, C-0 (1.3%) was only slightly detected on the surface of intact plate as in (A). The plates exposed to electron beams (50 KGy) (B) and to γ ray (100 KGy) showed C-0 (8.2%) and C-0 (5.3%), C = 0 (3.9%), indicating significant introduction of oxygen.

FIELD OF INDUSTRY APPLICABLE

According to the present invention, antibodies specific to antiphospholipid syndrome can be specifically assayed by using only β2-glycoprotein I. The present invention does not, therefore, require combination of β2-glycoprotein I and phospholipid for assaying antibodies specific to antiphospholipid syndrome, so that reagents for the assay can be prepared in an extremely simple manner.

Further, according to the present invention, antibodies specific to antiphospholipid syndrome can be assayed differentially from antibodies specific to infectious diseases, by using the solid phase reagent comprising a carrier having the surface on which functional groups containing a negative charge or a lone pair and/or free radicals having a negative charge or a lone pair have been previously introduced and having two sites on the surface, one of which has been coated with β2-glycoprotein and another has been coated with no β2-glycoprotein; or by using two solid phase reagents, one of which comprises a carrier having the surface on which the function groups and/or free radicals have been introduced and having β2-glycoprotein coated on the surface, and another comprises a carrier having the surface on which the functional groups and/or free radicals have been introduced and having no β2-glycoprotein coated on the surface.

Claims

1. A solid phase reagent comprising a carrier having the surface, onto which functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair have been introduced, and having β2-glycoprotein I coated on the surface.

2. A solid phase reagent according to claim 1, wherein said carrier is a synthetic resin exposed to radiations or electron beams thereby to have functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair introduced thereon.

3. A solid phase reagent according to claim 1, wherein said carrier is a synthetic resin treated with ozon or plasma thereby to have functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair introduced thereon.

4. A solid phase reagent according to claim 1, wherein said negative charge or said lone pair is derived from an oxygen atom.

5. A solid phase reagent according to claim 1, wherein said functional groups containing a negative charge or a lone pair are selected from hydroxy, carboxyl, carbonyl, formyl, imino, nitro, thiol and sulfonyl groups.

6. A solid phase reagent according to claim 1, wherein said free radicals containing a negative charge or a lone pair are oxygen radicals.

7. An assay method for antibodies specific to antiphospholipid syndrome which comprises using a solid phase reagent of claim 1.

8. A kit for use in assaying antibodies specific to antiphospholipid syndrome, comprising a solid phase reagent of claim 1 as a constituent reagent.

9. A solid phase reagent comprising a carrier having the surface, onto which functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair have been introduced, and having two sites, one of which being a site on which β2-glycoprotein I has been coated and another being a site on which no β2-glycoprotein I has been coated.

10. A method for detecting antibodies specific to antiphospholipid syndrome differentially from antibodies specific to infectious diseases, which comprises using a solid phase reagent of claim 9 and determining the reactivities of antibodies in a sample solution with the respective sites in said carrier of the solid phase reagent.

11. A kit for use in differentially detecting antibodies specific to an antiphospholipid syndrome from antibodies specific to infectious diseases, comprising a solid phase reagent of claim 9 as a constituent reagent.

12. A method for differentially detecting antibodies specific to an antiphospholipid syndrome from antibodies specific to infectious diseases, which comprises

using two solid phase reagents, one of which comprising a carrier having the surface onto which functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair have been introduced and having β2-glycoprotein I coated on the surface, and another comprising said carrier having no β2-glycoprotein I coated thereon, and

determining the reactivities of antibodies in a sample solution with said two solid phase reagents.

13. A kit for use in differentially detecting antibodies specific to antiphospholipid syndrome from antibodies specific to infectious diseases, which comprises

two solid phase reagents, one of which comprising a carrier having the surface onto which functional groups containing a negative charge or a lone pair and/or free radicals containing a negative charge or a lone pair have been introduced and having β2-glycoprotein I coated on the surface, and another comprising said carrier having no β2-glycoprotein I coated thereon.

FIG. 1

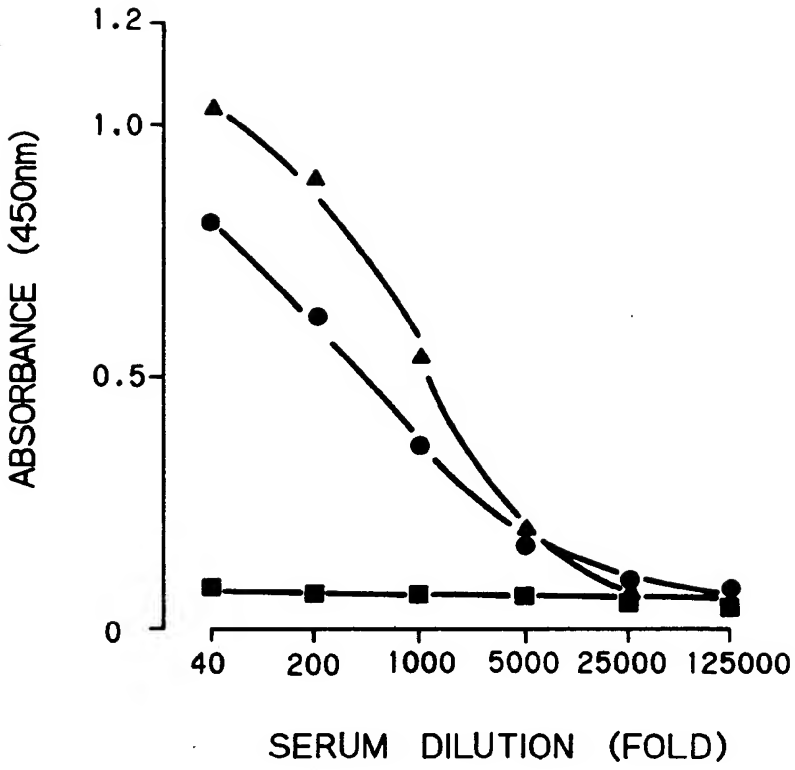

1.2
1.0
0.5
0
ABSORBANCE (450nm)
40
200
1000
5000
25000
125000
SERUM DILUTION (FOLD)

FIG. 2

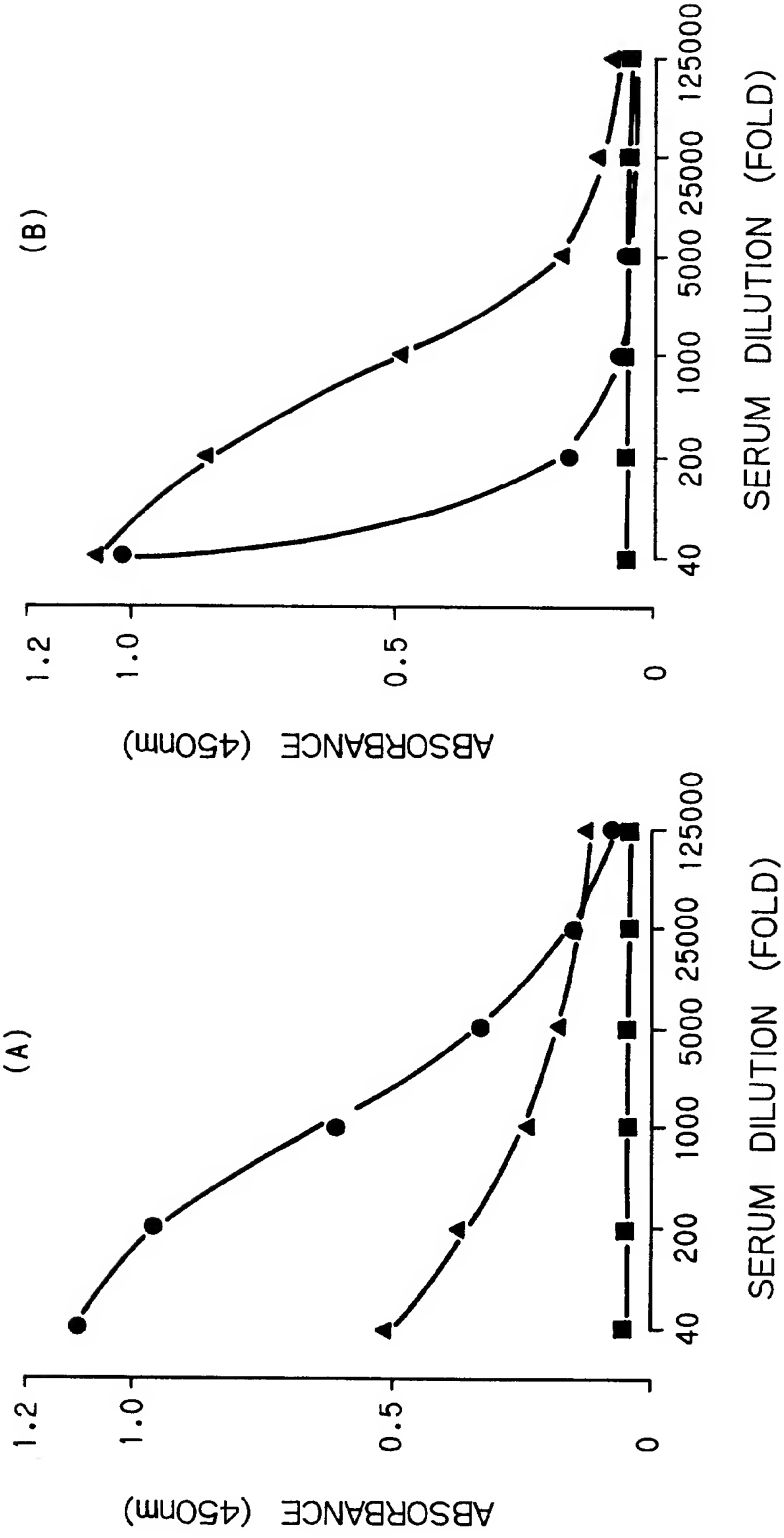

(A)
(B)
ABSORBANCE (450nm)
1.2
1.0
0.5
0
40
200
1000
5000
25000
125000
SERUM DILUTION (FOLD)

FIG. 3

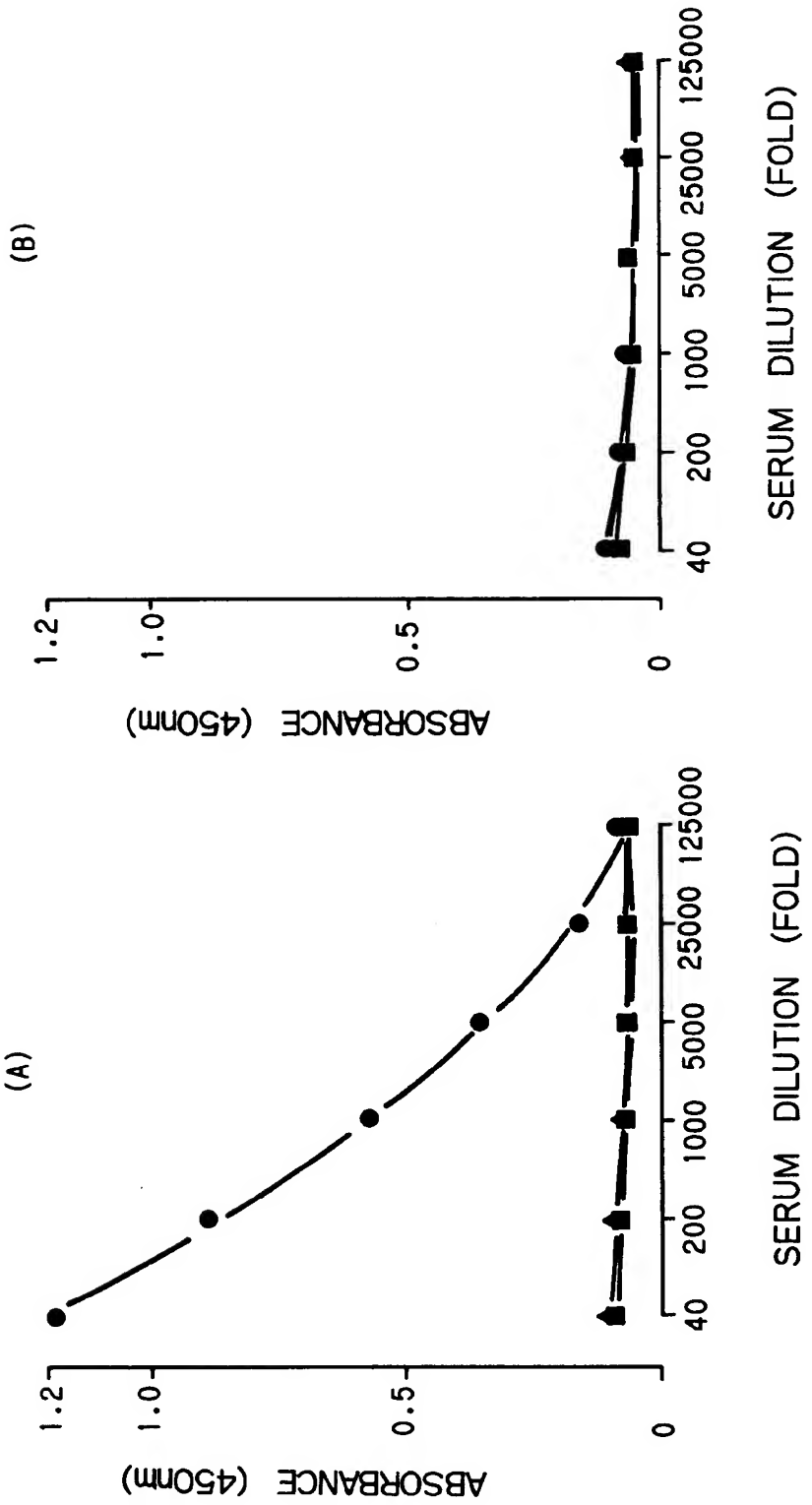

(A)
(B)
ABSORBANCE (450nm)
1.2
1.0
0.5
0
40
200
1000
5000
25000
125000
SERUM DILUTION (FOLD)

FIG. 4

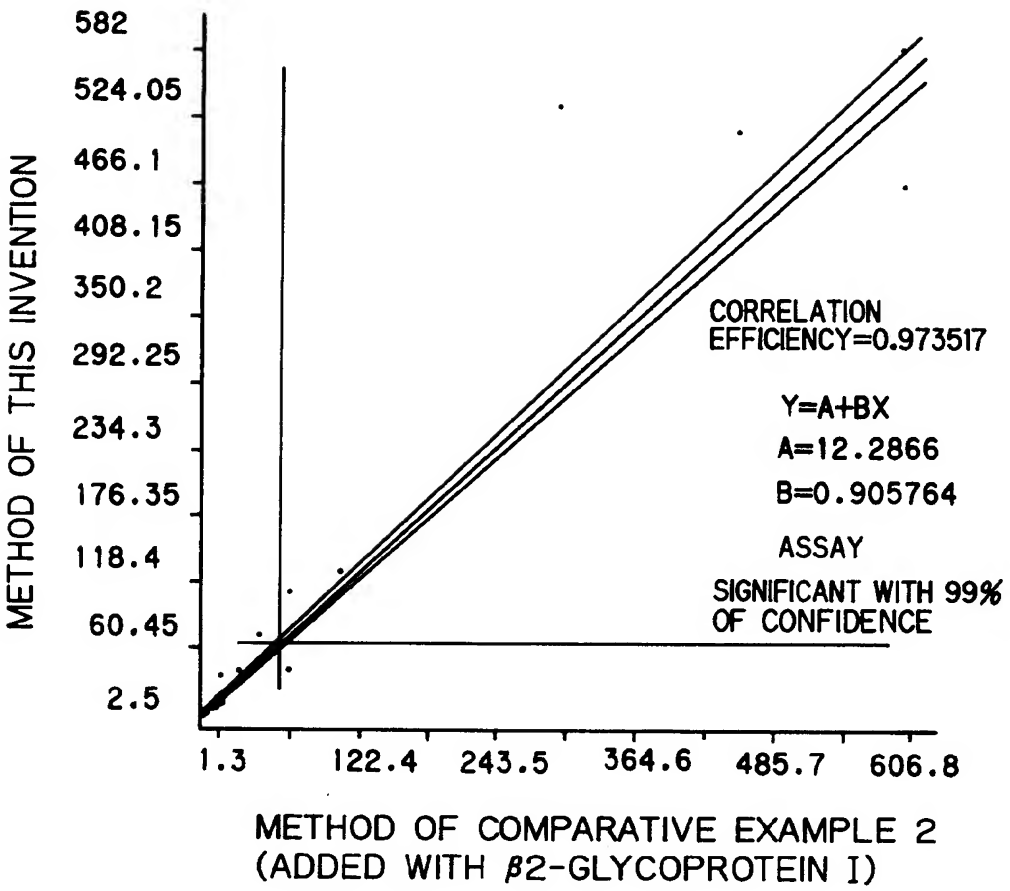

582
524.05
466.1
408.15
350.2
292.25
234.3
176.35
118.4
60.45
2.5
METHOD OF THIS INVENTION
1.3
122.4
243.5
364.6
485.7
606.8
METHOD OF COMPARATIVE EXAMPLE 2
(ADDED WITH β2-GLYCOPROTEIN I)
CORRELATION
EFFICIENCY=0.973517
Y=A+BX
A=12.2866
B=0.905764
ASSAY
SIGNIFICANT WITH 99%
OF CONFIDENCE

FIG. 5

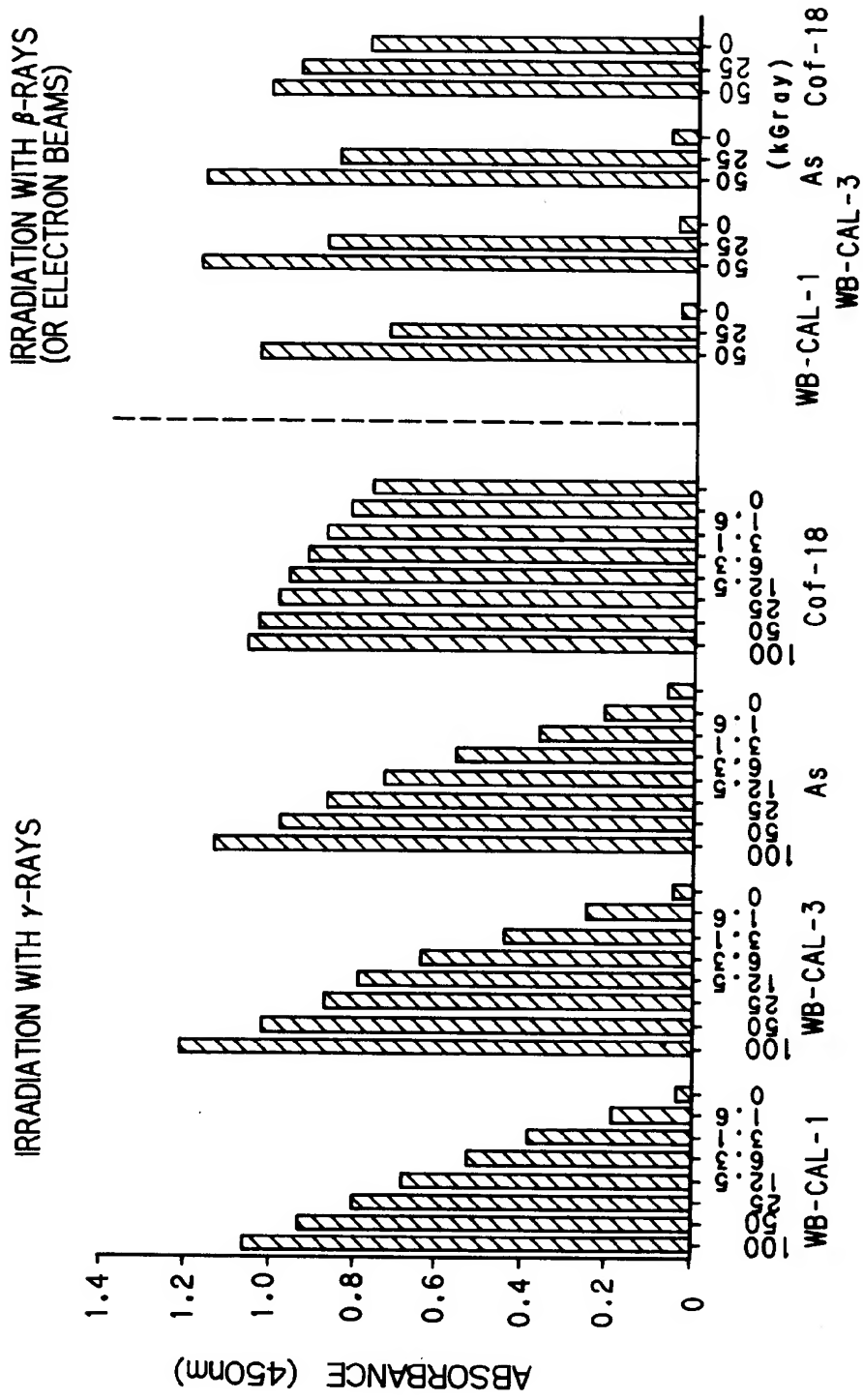

IRRADIATION WITH γ-RAYS
IRRADIATION WITH β-RAYS
(OR ELECTRON BEAMS)
ABSORBANCE (450nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
WB-CAL-1
WB-CAL-3
As
Cof-18
(kGray)

FIG. 6

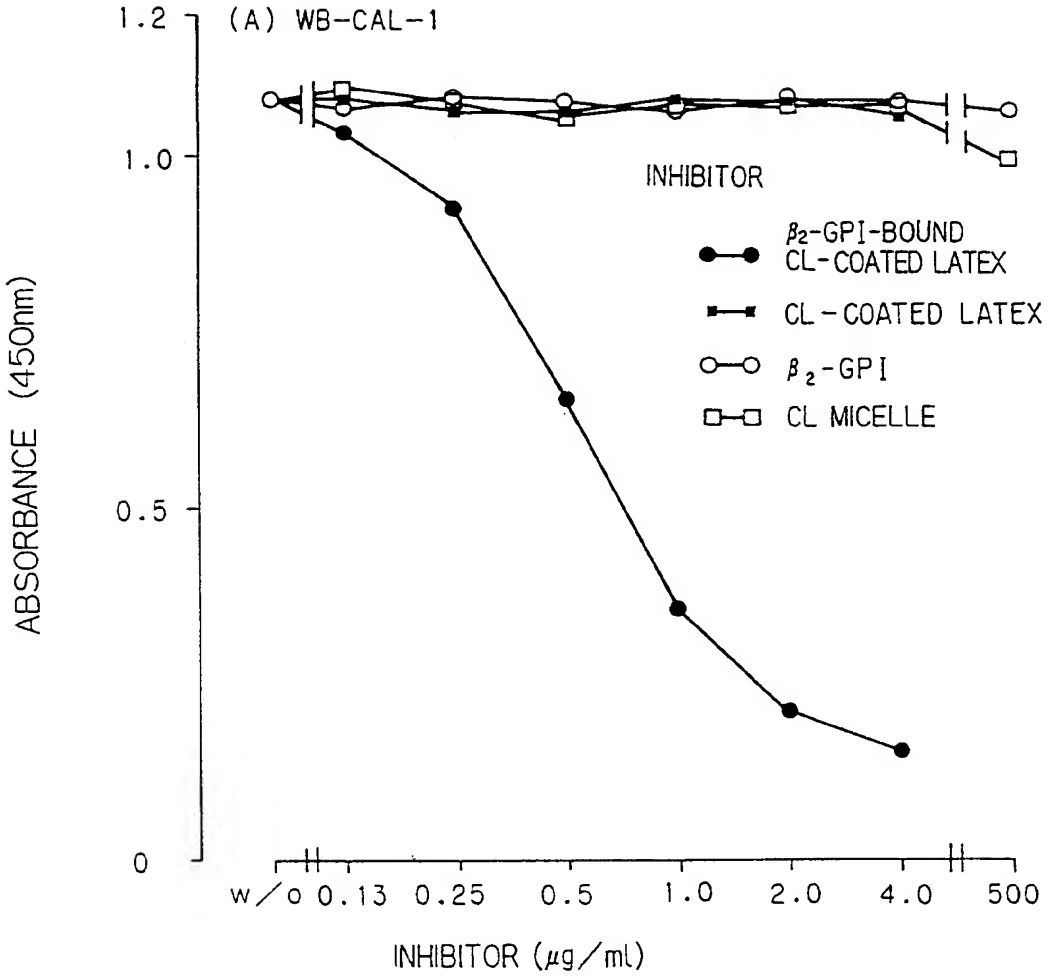

(A) WB-CAL-1
1.2
1.0
0.5
0
ABSORBANCE (450nm)
INHIBITOR
β2-GPI-BOUND CL-COATED LATEX
CL-COATED LATEX
β2-GPI
CL MICELLE
w/o 0.13 0.25 0.5 1.0 2.0 4.0 500
INHIBITOR (μg/ml)

FIG. 7

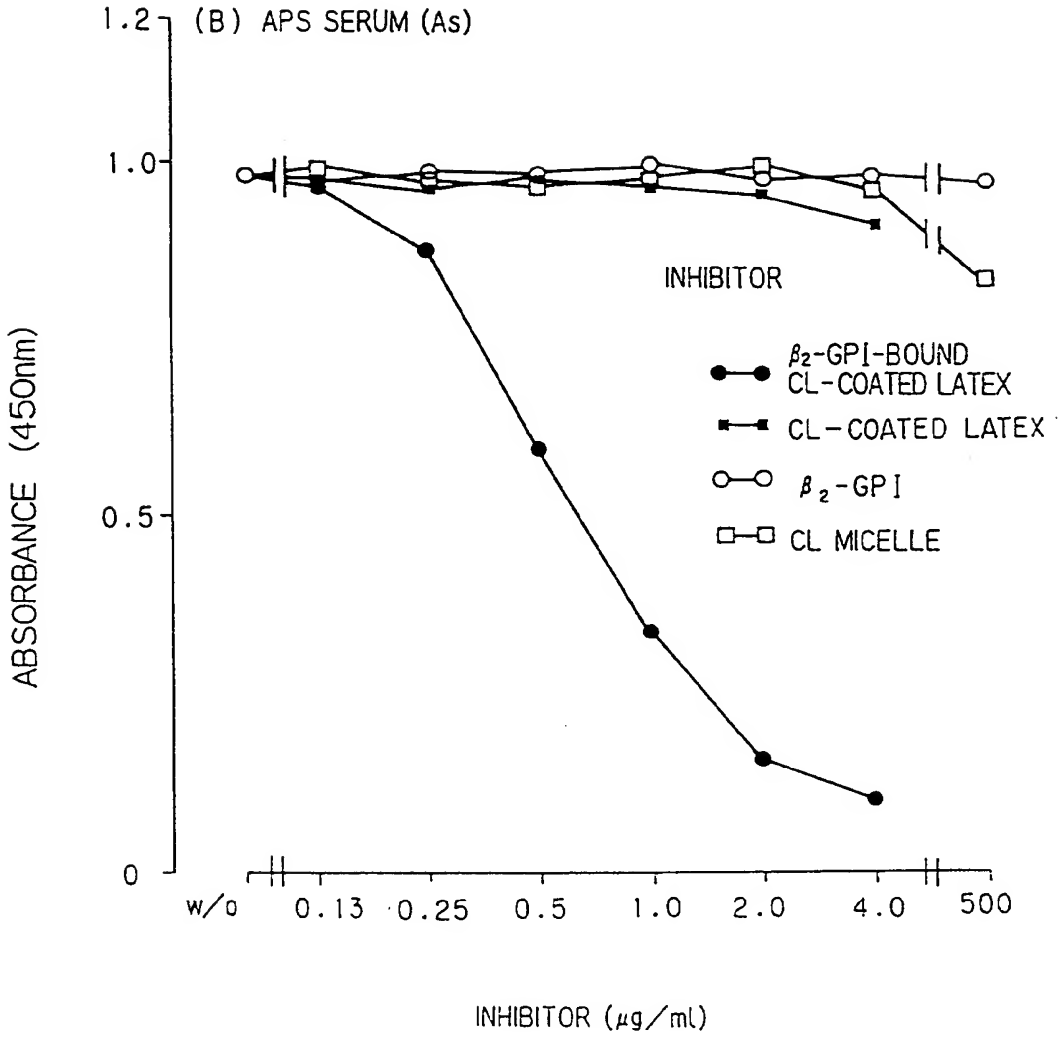

(B) APS SERUM (As)
1.2
1.0
0.5
0
ABSORBANCE (450nm)
INHIBITOR
β2-GPI-BOUND CL-COATED LATEX
CL-COATED LATEX
β2-GPI
CL MICELLE
w/o
0.13
0.25
0.5
1.0
2.0
4.0
500
INHIBITOR (μg/ml)

FIG. 8

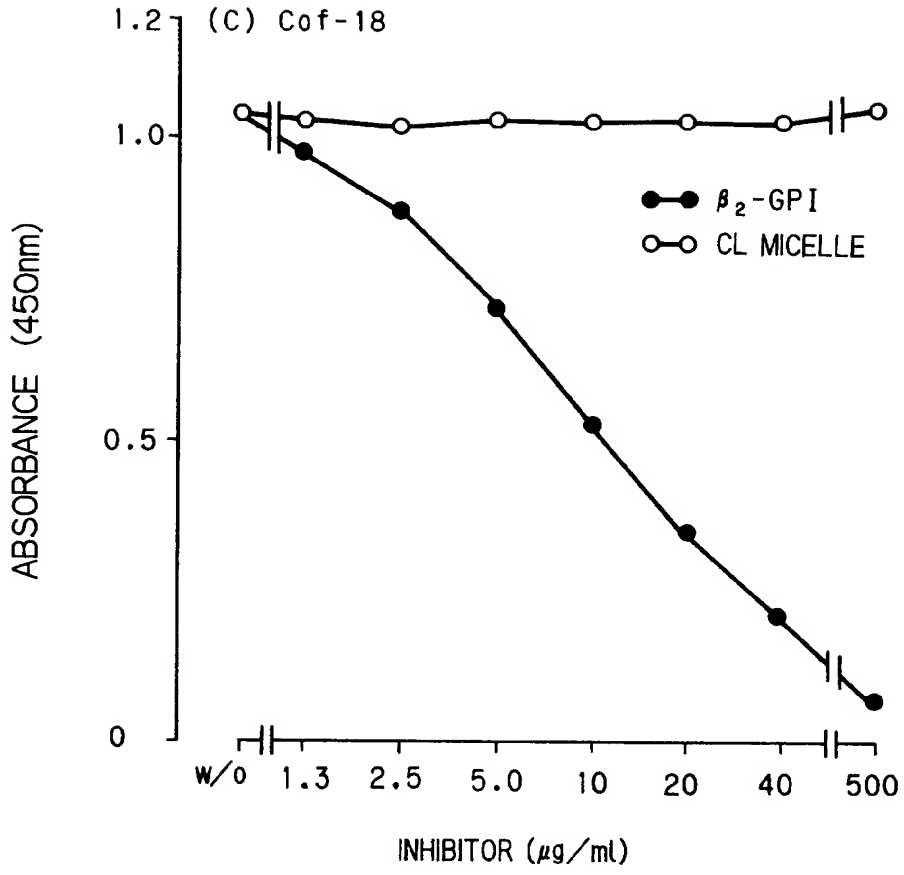

(C) Cof-18
1.2
1.0
0.5
0
ABSORBANCE (450nm)
β2-GPI
CL MICELLE
W/O
1.3
2.5
5.0
10
20
40
500
INHIBITOR (μg/ml)

FIG. 9

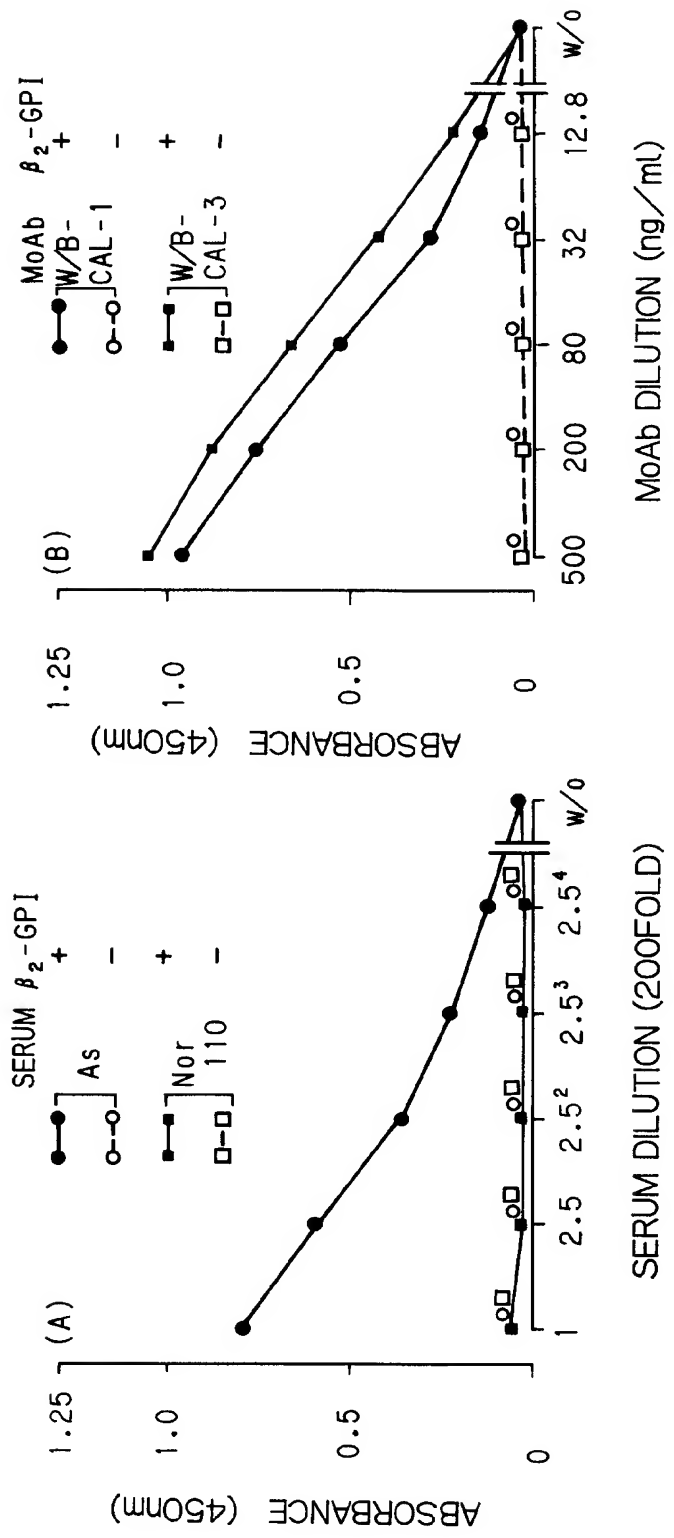

(A)
(B)
ABSORBANCE (450nm)
1.25
1.0
0.5
0
SERUM β2-GPI
+
−
As
Nor
110
1
2.5
2.5²
2.5³
2.5⁴
W/O
SERUM DILUTION (200FOLD)
MoAb β2-GPI
W/B-
CAL-1
CAL-3
500
200
80
32
12.8
MoAb DILUTION (ng/ml)

FIG. 10

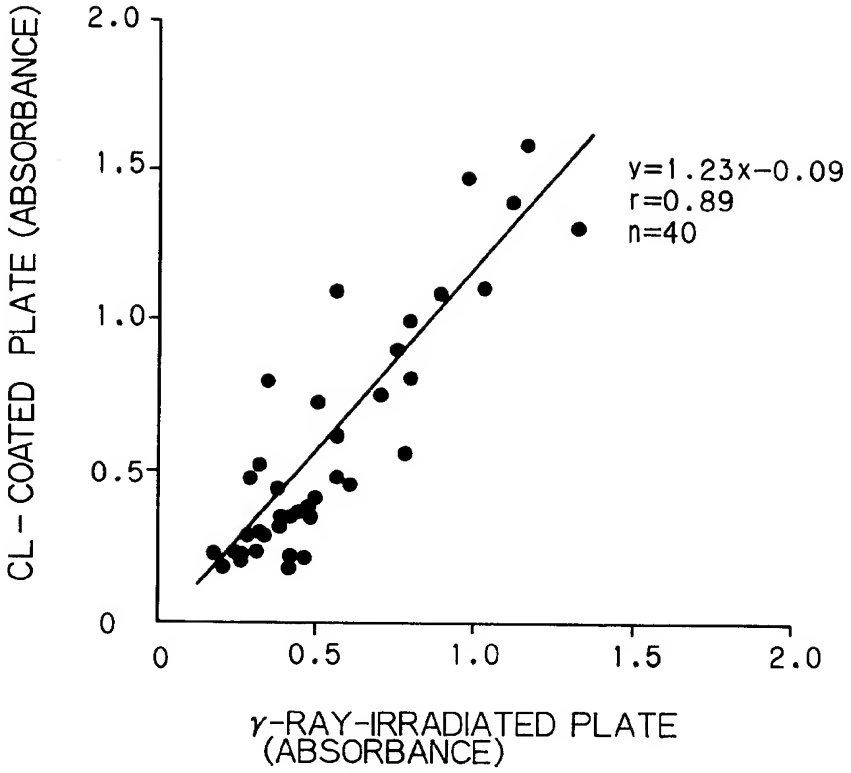

2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
CL-COATED PLATE (ABSORBANCE)
γ-RAY-IRRADIATED PLATE (ABSORBANCE)
y=1.23x-0.09
r=0.89
n=40

FIG. 11

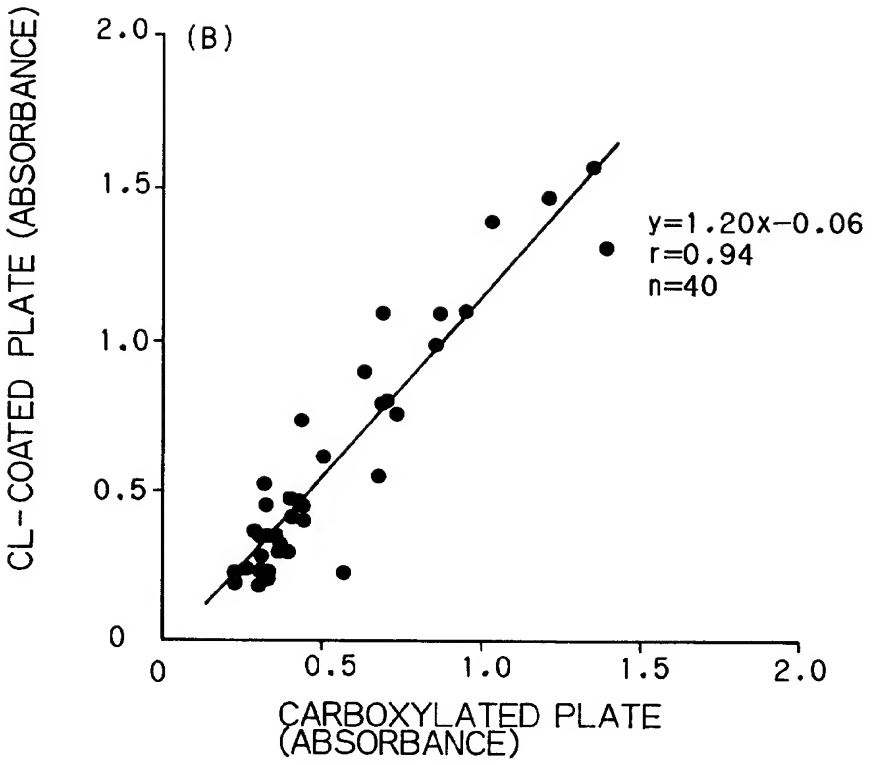

(B)
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
CL-COATED PLATE (ABSORBANCE)
CARBOXYLATED PLATE (ABSORBANCE)
y=1.20x-0.06
r=0.94
n=40

FIG. 12

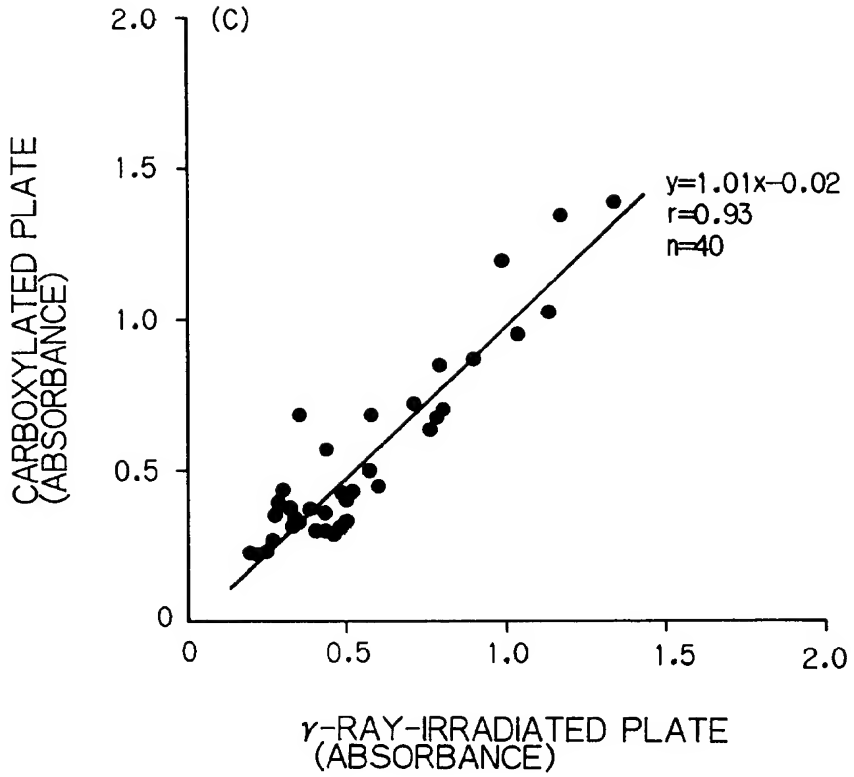

(C)
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
y=1.01x-0.02
r=0.93
n=40
CARBOXYLATED PLATE (ABSORBANCE)
γ-RAY-IRRADIATED PLATE (ABSORBANCE)

FIG. 13

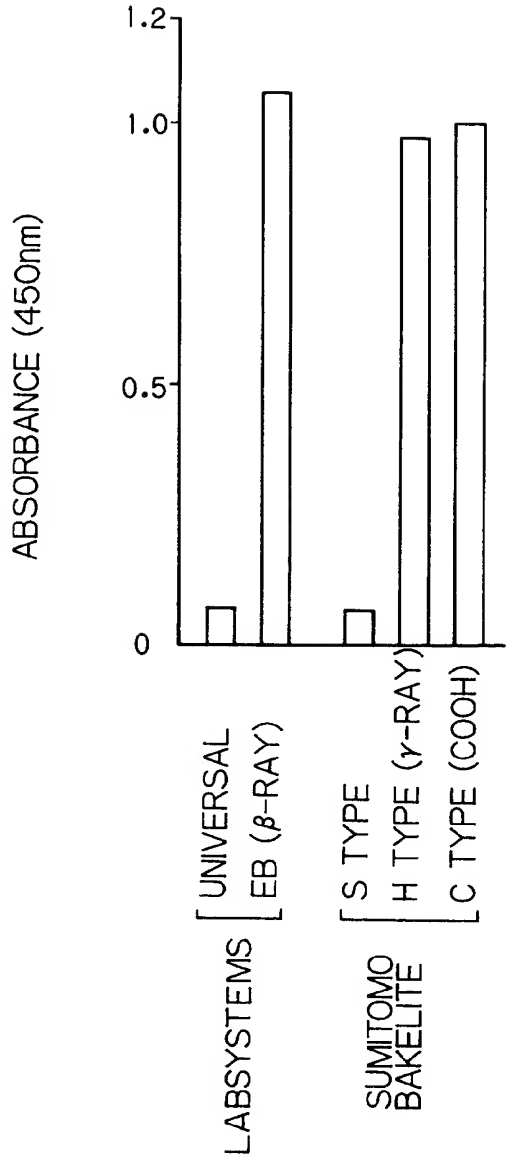

1.2
1.0
0.5
0
ABSORBANCE (450nm)
LABSYSTEMS
UNIVERSAL
EB (β-RAY)
SUMITOMO BAKELITE
S TYPE
H TYPE (γ-RAY)
C TYPE (COOH)

FIG. 14

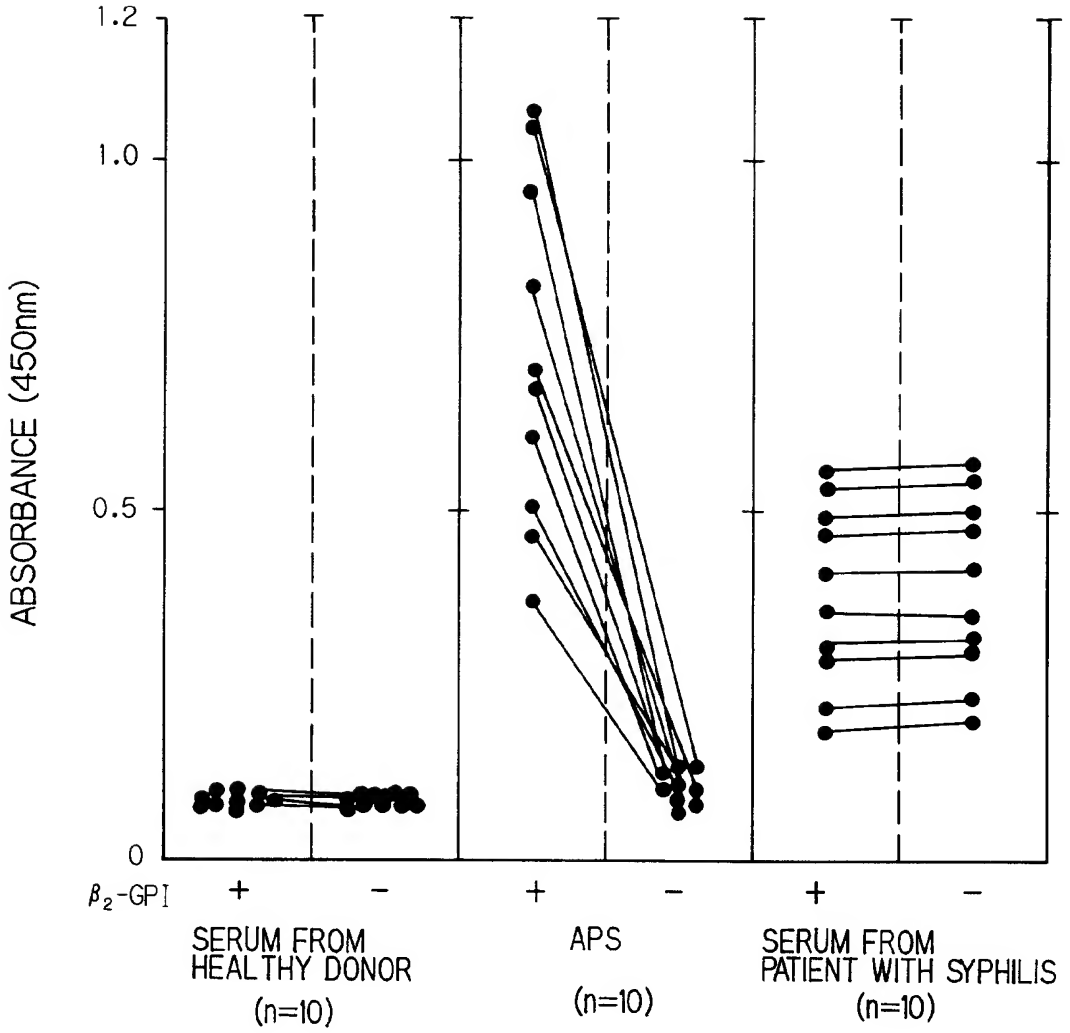
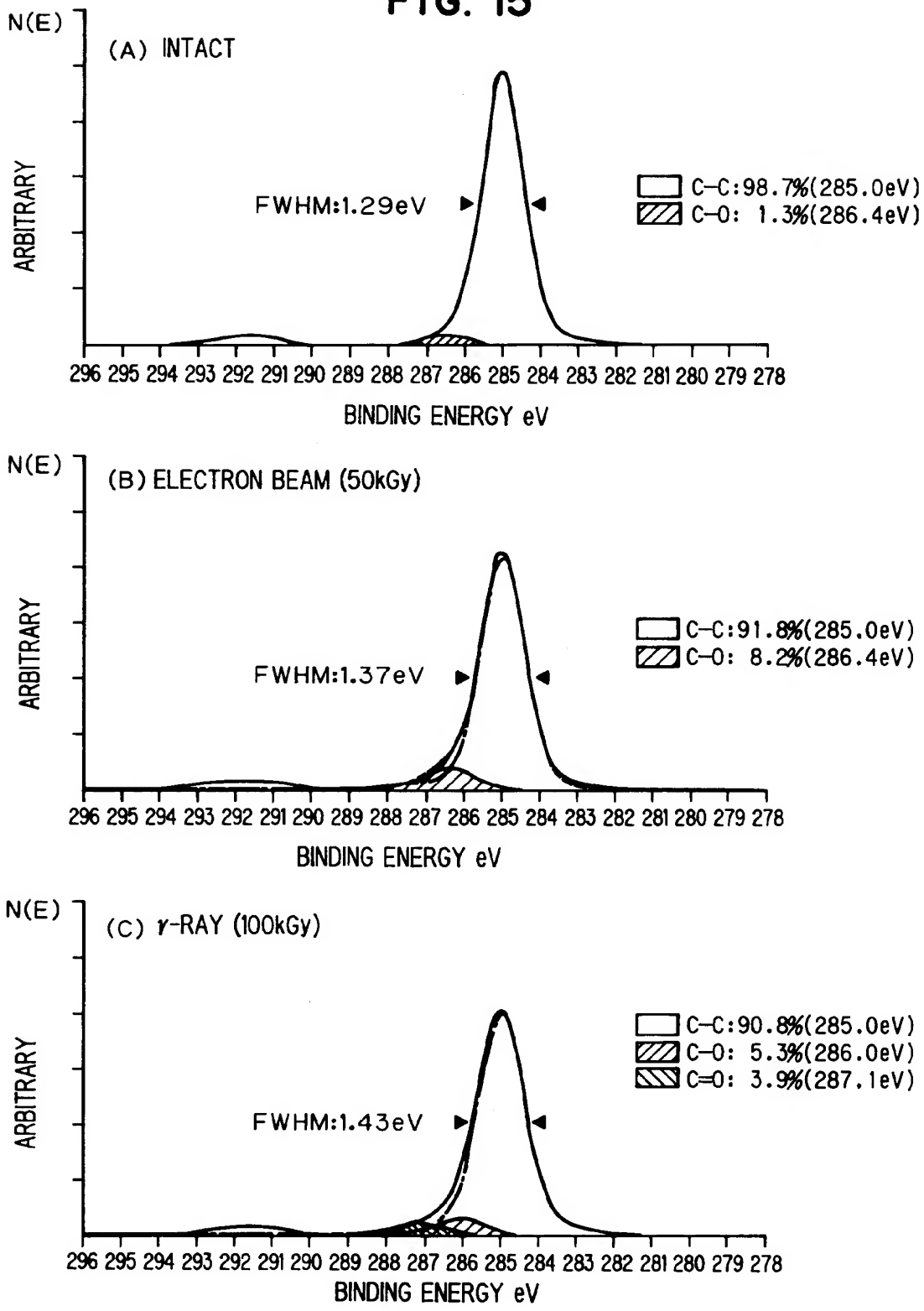

1.2
1.0
0.5
0
ABSORBANCE (450nm)
β2-GPI
+
−
+
−
+
−
SERUM FROM HEALTHY DONOR (n=10)
APS (n=10)
SERUM FROM PATIENT WITH SYPHILIS (n=10)

FIG. 15


N(E)
(A) INTACT
ARBITRARY
FWHM:1.29eV
C-C:98.7%(285.0eV)
C-O: 1.3%(286.4eV)
296 295 294 293 292 291 290 289 288 287 286 285 284 283 282 281 280 279 278
BINDING ENERGY eV
N(E)
(B) ELECTRON BEAM (50kGy)
ARBITRARY
FWHM:1.37eV
C-C:91.8%(285.0eV)
C-O: 8.2%(286.4eV)
296 295 294 293 292 291 290 289 288 287 286 285 284 283 282 281 280 279 278
BINDING ENERGY eV
N(E)
(C) γ-RAY (100kGy)
ARBITRARY
FWHM:1.43eV
C-C:90.8%(285.0eV)
C-O: 5.3%(286.0eV)
C=O: 3.9%(287.1eV)
296 295 294 293 292 291 290 289 288 287 286 285 284 283 282 281 280 279 278
BINDING ENERGY eV

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP93/00144

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl^5 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl^5 G01N33/53-33/579

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho 1926 - 1992
Kokai Jitsuyo Shinan Koho 1971 - 1992

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 91/15772 (Yamasa Shoyu Co., Ltd.), October 17, 1991 (17. 10. 91), & AU, A, 9176678 & EP, A, 474849 & JP, A, 4506415	1-13
X	Journal of Immunological Methods, Vol. 143, No. 2, 1991, J. Arvieux et al.: "Measurement of anti-phospholipid antibodies by ELISA using Beta-2 Glycoprotein I as an antigen", p. 223-230	1-13
A	American Journal of Medicine, Vol. 93, No. 2, 1992, J. P. Viard et al.: "Association of anti-Beta-2 Glycoprotein I antibodies with lupus-type circulating anticoagulant and thrombosis in systemic lupus erythematosus", p. 181-186	1-13
A	JP, A, 2-304364 (Abbot Laboratories), December 18, 1990 (18. 12. 90), Claim, & EP, A, 396116 & CA, A, 2015938	1, 4, 5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
March 9, 1993 (09. 03. 93)	April 13, 1993 (13. 04. 93)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP93/00144

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, A, 1-223352 (General Biometrics Inc.), September 6, 1989 (06. 09. 89), Line 9, upper right column to line 3, lower left column, page 4 & EP, A, 324603	1, 4, 5
A	JP, A, 63-18268 (Olympus Optical Co., Ltd.), January 26, 1988 (26. 01. 88), Claim (Family: none)	1, 4, 5
A	JP, A, 3-72261 (Nitto Denko K.K.), March 27, 1991 (27. 03. 91), Claim (Family: none)	1, 4, 5
A	JP, A, 62-123359 (Sumitomo Bakelite Co., Ltd.), June 4, 1987 (04. 06. 87), Claim (Family: none)	3
A	JP, A, 60-91983 (Shionogi & Co., Ltd.), May 23, 1985 (23. 05. 85), Line 20, lower left column, page 2 to line 3, upper left column, page 3 & EP, A, 141627 & GB, A, 2148905	3, 4, 6
A	JP, A, 2-71152 (Terumo Corp.), March 9, 1990 (09. 03. 90), Claim (Family: none)	3
A	JP, A, 2-131500 (Industrial Technology Research Institute), May 21, 1990 (21. 05. 90), Claim & EP, A, 351950 & US, A, 5028657 & US, A, 5171779	3, 6
A	JP, A, 59-58004 (Japan Atomic Energy Research Institute), April 3, 1984 (03. 04. 84), Lines 6 to 10, lower right column, page 3 & US, A, 4552633	2
A	JP, A, 60-222774 (Bio-Rink Aktieverted Polymaies AV.), November 7, 1985 (07. 11. 85), Claim & EP, A, 155252	1, 2, 4, 5
A	JP, A, 60-260857 (Sumitomo Bakelite Co., Ltd.), December 24, 1985 (24. 12. 85), Claim (Family: none)	2
A	JP, A, 3-128461 (Cansar Diagnostics, Inc.), May 31, 1991 (31. 05. 91), Claim & US, A, 5016644 & US, A, 5133363 & EP, A, 509158 & CA, A, 2040088	9-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP93/00144

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, A, 2-504550 (Darurgar Riilave), December 20, 1990 (20. 12. 90), Lines 4 to 15, lower right column, page 3 & WO, A, 8901155 & EP, A, 382734	9-13
A	JP, A, 59-15861 (Konica Corp.), January 26, 1984 (26. 01. 84), Claim (Family: none)	9-13